Xylitol Inhibits Inflammatory Cytokine Expression Induced by Lipopolysaccharide from *Porphyromonas gingivalis*

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Porphyromonas gingivalis* is one of the suspected periodontopathic bacteria. The lipopolysaccharide (LPS) of *P. gingivalis* is a key factor in the development of periodontitis. Inflammatory cytokines play important roles in the gingival tissue destruction that is a characteristic of periodontitis. Macrophages are prominent at chronic inflammatory sites and are considered to contribute to the pathogenesis of periodontitis. Xylitol stands out and is widely believed to possess anticaries properties. However, to date, little is known about the effect of xylitol on periodontitis. The aim of the present study was to determine tumor necrosis factor alpha (TNF-α) and interleukin-1β (IL-1β) expression when RAW 264.7 cells were stimulated with *P. gingivalis* LPS (hereafter, LPS refers to *P. gingivalis* LPS unless stated otherwise) and the effect of xylitol on the LPS-induced TNF-α and IL-1β expression. The kinetics of TNF-α and IL-1β levels in culture supernatant after LPS treatment showed peak values at 1 h (TNF-α) and 2 to 4 h (IL-1β), respectively. NF-κB, a transcription factor, was also activated by LPS treatment. These cytokine expressions and NF-κB activation were suppressed by pretreatment with pyrrolidine dithiocarbamate (an inhibitor of NF-κB). Pretreatment with xylitol inhibited LPS-induced TNF-α and IL-1β gene expression and protein synthesis. LPS-induced mobilization of NF-κB was also inhibited by pretreatment with xylitol in a dose-dependent manner. Xylitol also showed inhibitory effect on the growth of *P. gingivalis*. Taken together, these findings suggest that xylitol may have good clinical effect not only for caries but also for periodontitis by its inhibitory effect on the LPS-induced inflammatory cytokine expression.

Periodontal disease comprises a group of infections that leads to inflammation of the gingival tissues and destruction of periodontal tissues and, in severe cases, is accompanied by the loss of alveolar bone with eventual exfoliation of the teeth (28). Periodontitis results from infection with subgingival plaque-forming bacteria, followed by host immune responses. Although subgingival dental plaque is a complex microbial community in which more than 300 different species have been identified, a consensus view on the major potential periodontopathogens has emerged, and *Porphyromonas gingivalis* is now regarded to be one of the most important of these bacteria (20). *P. gingivalis*, a gram-negative anaerobic bacterium found in periodontal pocket, has been implicated as an important pathogen that plays a role in the initiation and progression of periodontitis (14, 37) and expresses numerous potential virulence factors.

Even though periodontal destruction is partly caused by proteinases secreted from bacteria, it is now accepted that host response to such bacterial products is the major cause of the pathogenesis (4, 15). Products of *P. gingivalis*, such as lipopolysaccharide (LPS), membrane proteins, and bacterial proteinases, are known to be able to induce the response of local cells for secreting high level of several cytokines that lead to periodontal tissue destruction (4). LPS is a major component of the outer membrane of gram-negative bacteria (42). Proinflammatory cytokines such as interleukin-1 (IL-1) and tumor necrosis factor alpha (TNF-α) initiate and augment subsequent inflammatory cascades, leading to tissue destruction (44). *P. gingivalis* LPS (hereafter, LPS refers to *P. gingivalis* LPS unless stated otherwise) differs from the LPS of enterobacterial species in its structural and functional properties (19, 29). It has been reported that LPS stimulates the induction of IL-1α, IL-1β, IL-6, and IL-8 by human gingival fibroblasts (31).

Xylitol stands out and is widely believed to possess anticaries properties. This fact indicates xylitol's superiority over the other sugar alcohols for potential caries control (reviewed in references 40 and 43). Xylitol is a polyol sugar alcohol and is referred to as birch sugar because it can be produced from birch. Natural sources of xylitol include plums, strawberries, raspberries, and rowanberries (26). The inhibitory effect of xylitol on the growth of *Streptococcus mutans*, one of primary causative agents of dental caries has been reported (7, 35, 45). However, little is known about the effect of xylitol on the growth of *P. gingivalis* or on periodontal disease.

Macrophages are prominent at chronic inflammatory sites, including periodontal disease, and are considered to contribute to the pathogenesis of the disease. Therefore, in the present study we determined the expression of proinflammatory cytokines such as TNF-α and IL-1β when mouse macrophage cell line, RAW 264.7 cells were stimulated with LPS. The effects of xylitol on the LPS-induced TNF-α and IL-1β expression and on the growth of *P. gingivalis* were also examined.

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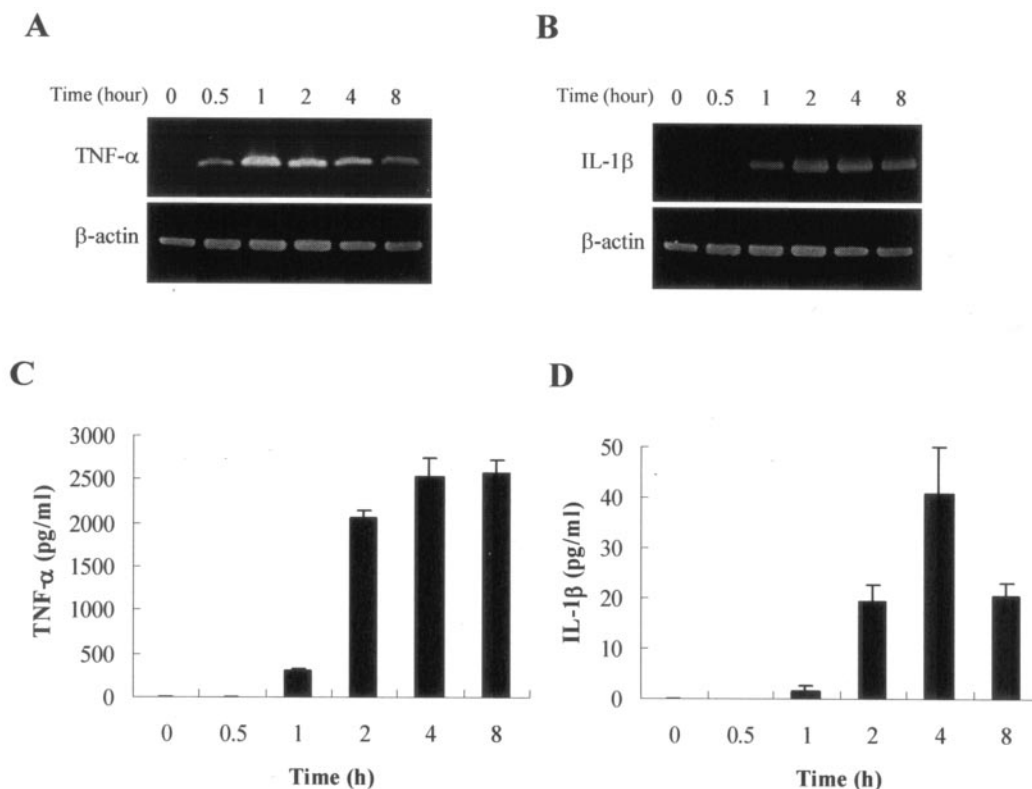
A   B

FIG. 1. (A and B) Time kinetics of LPS-induced TNF-α (A) and IL-1β (B) mRNA expression. RAW 264.7 cells were treated with LPS (1 μg/ml), and total RNA was prepared at the times indicated. RT-PCR was performed as described in Materials and Methods. (C and D) Time kinetics of LPS-induced TNF-α (C) and IL-1β (D) protein production. RAW 264.7 cells were treated with 1 μg of LPS/ml, and the culture supernatants were collected at the times indicated. The levels of cytokine production in culture supernatants were measured by ELISA. Values are expressed as mean $\pm$ the SD obtained from three independent experiments.

MATERIALS AND METHODS

Reagents. Mouse TNF-α and IL-1β enzyme-linked immunosorbent assay (ELISA) kits were purchased from R&D Systems (Minneapolis, MN). Pyrrolidine dithiocarbamate (PDTC) was purchased from Calbiochem (San Diego, CA).

Bacterial culture. *P. gingivalis* A7A1-28 was cultured in brain heart infusion broth, which contained 5 mg of hemin and 0.5 mg of vitamin K/ml at 37°C in an anaerobic chamber in an atmosphere containing 90% N_2, 5% H_2, and 5% CO_2.

LPS purification. *P. gingivalis* was grown under anaerobic conditions and harvested at the end of the logarithmic phase of growth. LPS extraction was achieved by the hot phenol-water method (47). Briefly, the bacterial cell pellet was suspended in pyrogen-free water, and then an equal volume of 90% phenol at 60°C was added dropwise for 20 min and stirred constantly. The aqueous phase was separated by centrifugation at 7,000 rpm for 15 min at 4°C and collected. This process was repeated, and the aqueous phase was pooled and dialyzed against deionized water for 3 days at 4°C. The dialyzed LPS preparation was then centrifuged at 40,000 rpm for 1.5 h at 4°C in a Beckman (Palo Alto, CA) ultracentrifuge. The precipitate was suspended with 30 ml of pyrogen-free water, dialyzed against distilled water for 3 days, lyophilized, and stored at 4°C. LPS samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and stained for protein with Coomassie blue to confirm the purity of the LPS moieties.

Cell culture. The mouse macrophage cell line RAW 264.7 was purchased from American Type Culture Collection (Rockville, MD). Cells were maintained in Dulbecco modified Eagle medium with 10% fetal bovine serum (Life Technologies, Inc., Paisley, Scotland), 100 U of penicillin/ml, and 100 μg of streptomycin/ml and were incubated at 37°C in a humidified atmosphere of 5% CO_2.

RT-PCR. Total RNA was prepared from RAW 264.7 cells by using a reagent (TRIzol; Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The concentration of the RNA obtained was determined by measuring the absorbance at 260 and 280 nm. Total RNA (1 μg) isolated from each sample was

used as a template for the cDNA synthesis. The reverse transcription (RT) of total RNA to cDNA was performed by using AccuPower RT PreMix (Bioneer Co., Daejeon, Korea). cDNA was amplified by PCR with a Perkin-Elmer thermal cycler (denaturation for 30 s at 95°C, annealing for 30 s at 60°C, and elongation for 30 s at 72°C) with TNF-α (23 cycles), IL-1β (30 cycles), or β-actin primers (25 cycles). The primers used in these analysis were as follows: TNF-α, 5'-CCT GTAGCCCACGTCGTAGC-3' and 5'-TTGACCTCAGCGCTGAGTTG-3'; IL-1β, 5'-GATACAAACTGATGAAGCTCGTCA-3' and 5'-GAGATAGTG TTTGTCCACATCCTGA-3'; and β-actin, 5'-GGGTCAGAACTCCTATG-3' and 5'-GTAACAATGCCATGTTCAAT-3'. A total of 20 μl of the RT-PCR products were separated in 1.2% (wt/vol) agarose gels and stained with ethidium bromide.

Measurement of TNF-α and IL-1β. The amounts of TNF-α and IL-1β released to the culture media after LPS stimulation were analyzed by using an ELISA kit from R&D systems (Minneapolis, MN). Briefly, standard or sample solution was added to an ELISA well plate, which had been precoated with specific monoclonal capture antibody. After incubation for 2 h at room temperature, polyclonal anti-TNF-α or anti-IL-1β antibody conjugated with horseradish peroxidase was added to the solution and incubated for 2 h at room temperature. Substrate solution containing hydrogen peroxidase and chromogen was added and allowed to react for 30 min. The levels of cytokine expression were assessed by ELISA reader at 450 nm. Each densitometric value expressed as mean $\pm$ the standard deviation (SD) was obtained from three independent experiments.

Nuclear extract preparation and gel shift assay. The nuclear extracts were prepared from RAW 264.7 cells according to a modification of the method of Dignam et al. (12). Briefly, cells were washed with ice-cold phosphate-buffered saline and pelleted. The cell pellet was resuspended in hypotonic buffer (10 mM HEPES [pH 7.9 at 4°C], 0.5 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM dithiothreitol [DTT], 0.2 mM phenylmethylsulfonyl fluoride [PMSF]) and incubated for 10 min on ice; the cells were then lysed by the addition of 10% IGEPAL CA-630 (Sigma Chemical Co., St. Louis, MO), followed by vigorous vortexing for 10 s. Nuclei
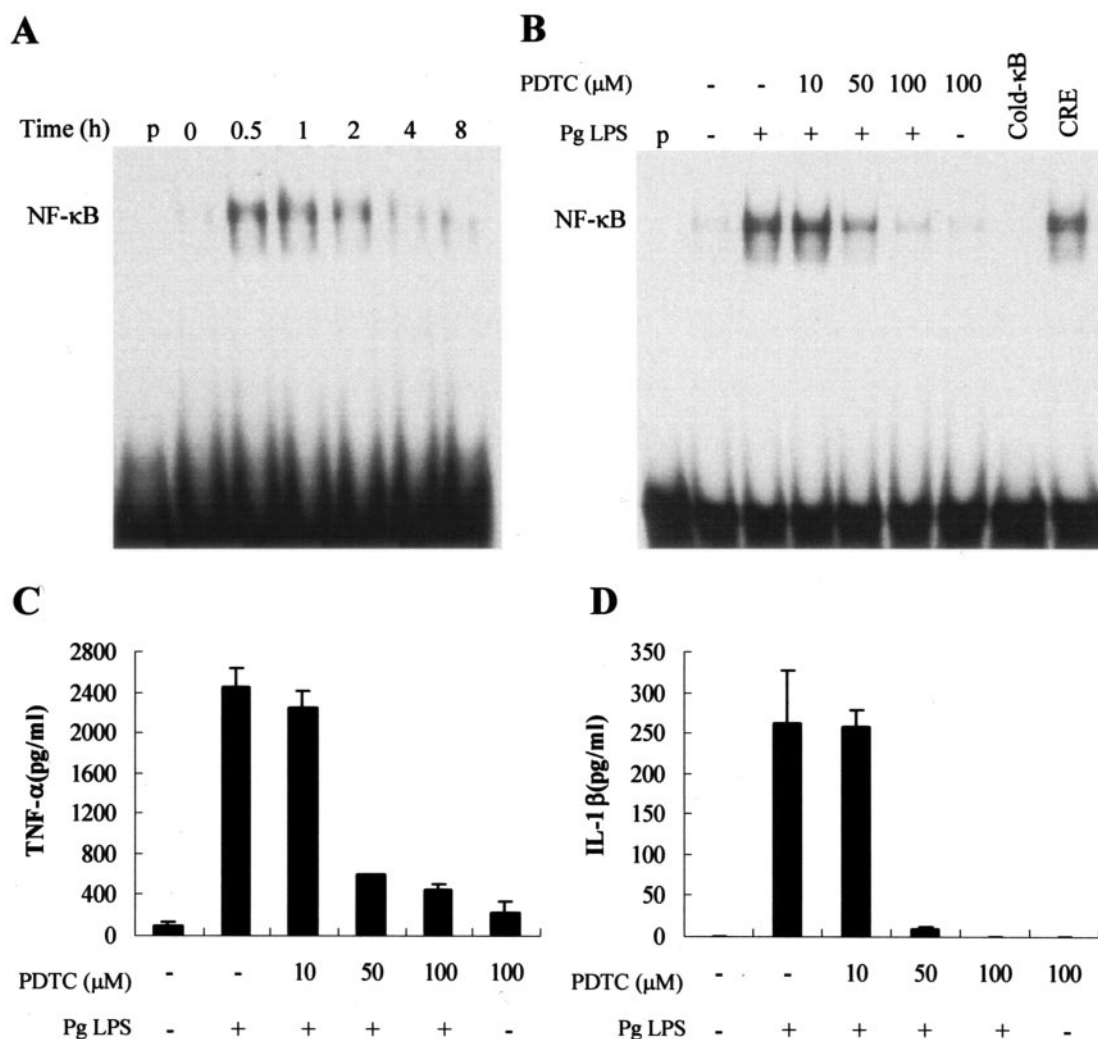
FIG. 2. (A) LPS-induced NF-κB activation in RAW 264.7 cells. These cells were stimulated with 1 μg of *P. gingivalis* LPS/ml for various time intervals. Nuclear extracts from RAW 264.7 cells after LPS treatment were incubated with α-^{32}P-labeled κB oligonucleotide and electrophoresed on a 4% polyacrylamide gel. Lane p contained probe incubated without extract. (B) Effects of PDTC on the LPS-induced NF-κB activation. RAW 264.7 cells were pretreated with various concentrations of PDTC for 30 min prior to treatment with 1 μg of LPS/ml. Nuclear extracts were prepared 30 min after LPS treatment, and a gel shift assay was performed. Lane p contained probe incubated without extract. A 50-fold excess of cold κB or cAMP response element oligonucleotide was added as competitor. (C and D) Inhibition of TNF-α (C) and IL-1β (D) expression by PDTC. The cells were pretreated with PDTC (10, 50, or 100 μM) for 30 min prior to treatment with 1 μg of LPS/ml. Culture supernatants were collected 4 h after LPS treatment, and the production of TNF-α and IL-1β was measured by ELISA. Values are expressed as mean ± the SD obtained from three independent experiments.

were pelleted and resuspended in low-salt buffer (20 mM HEPES [pH 7.9 at 4°C], 25% glycerol, 1.5 mM MgCl$_2$, 20 mM KCl, 0.2 mM EDTA, 0.5 mM dithiothreitol, 0.2 mM PMSF) and added high-salt buffer (20 mM HEPES [pH 7.9 at 4°C], 1.5 mM MgCl$_2$, 0.8 M KCl, 0.2 mM EDTA, 0.5 mM DTT, 0.2 mM PMSF) in a dropwise fashion. After 30 min of incubation at 4°C, the lysates were centrifuged, and supernatants containing the nuclear proteins were transferred to new vials. To inhibit endogenous protease activity, 1 mM PMSF was added. As a probe for the gel shift assay, an oligonucleotide containing the immunoglobulin κ-chain binding site (κB, 5′-CCGGTTAACAGAGGGGGC TTTCCGAG-3′) was synthesized. The two complementary strands were annealed and labeled with [α-^{32}P]dCTP. Labeled oligonucleotides (10,000 cpm), 10 μg of nuclear extracts, and binding buffer [10 mM Tris-HCl (pH 7.6), 500 mM KCl, 10 mM EDTA, 50% glycerol, 100 ng of poly(dI-dC), 1 mM DTT] were incubated for 30 min at room temperature in a final volume of 20 μl. The reaction mixture was analyzed by electrophoresis on a 4% polyacrylamide gel in 0.5× Tris-borate buffer. Specific binding was controlled by competition with a 50-fold excess of cold κB or cyclic AMP response element oligonucleotide.

RESULTS

***P. gingivalis* LPS-induced gene expression and protein synthesis of TNF-α and IL-1β.** TNF-α is a proinflammatory cytokine and has been implicated as having an important functional role in the bone and tissue destruction in periodontitis. To clarify whether *P. gingivalis* LPS can induce TNF-α and IL-1β gene expression in mouse macrophage cell line, RAW 264.7 cells, these cells were treated with 1 μg of LPS/ml, and total RNA was prepared at 30 min, 1 h, 2 h, 4 h, and 8 h after LPS treatment. The time kinetics of TNF-α and IL-1β mRNA expression was examined by RT-PCR. The TNF-α mRNA could be detected 30 min after LPS treatment and reached its maximum at 1 h and subsequently declined thereafter
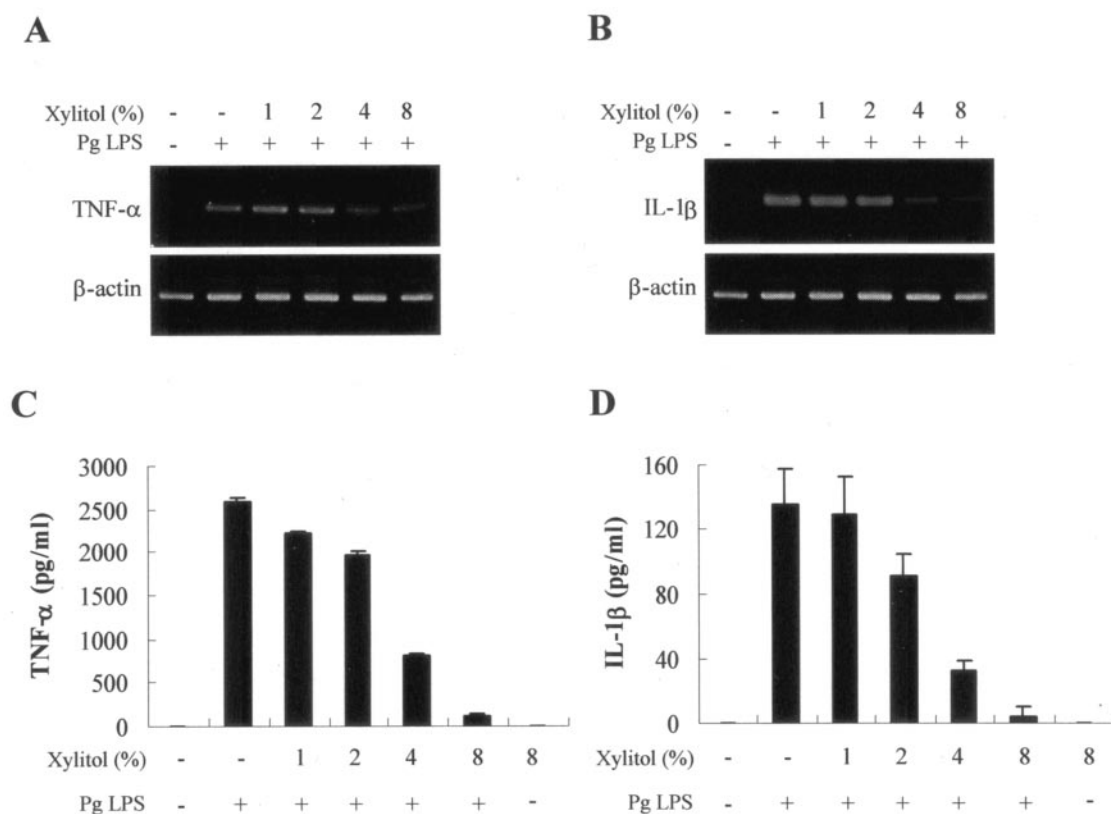
FIG. 3. (A and B) Effects of xylitol on LPS-induced TNF-α (A) and IL-1β (B) mRNA expression. RAW 264.7 cells were pretreated with various concentrations of xylitol 30 min prior to treatment with 1 μg of LPS/ml. Total RNA was prepared 1 and 4 h after LPS treatment, and RT-PCR was performed. (C and D) Effects of xylitol on LPS-induced TNF-α (C) and IL-1β (D) protein production. RAW 264.7 cells were pretreated with various concentrations of xylitol 30 min prior to treatment with 1 μg of LPS/ml. Culture supernatants were collected 4 h after LPS treatment, and the production of TNF-α and IL-1β was measured by ELISA. Values are expressed as means ± the SD obtained from three independent experiments.

(Fig. 1A). The time kinetics of IL-1β mRNA expression after LPS treatment showed peak values at 2 to 4 h (Fig. 1B). RAW 264.7 cells were treated with LPS, culture supernatants were collected at the indicated time points, and the protein production of TNF-α and IL-1β was measured by ELISA. TNF-α production was evident 1 h after LPS treatment and was increased gradually (Fig. 1C). IL-1β levels reached a peak 4 h after LPS treatment (Fig. 1D). These data indicate that *P. gingivalis* LPS can induce mRNA expression and protein production of TNF-α and IL-1β in the mouse macrophage cell line RAW 264.7.

NF-κB mobilization by LPS. NF-κB, a transcription factor, has a role in regulation of gene expression of proinflammatory cytokines. To elucidate a possible involvement of NF-κB in LPS-induced TNF-α and IL-1β gene expression, the effect of LPS on the activation of NF-κB in RAW 264.7 cells was investigated. For this purpose, a gel shift assay was conducted. When the cells were treated with 1 μg of LPS/ml for various time intervals, LPS markedly enhanced the binding of nuclear protein to the consensus sequences of the binding site for NF-κB. It was reached a maximal level of binding at 30 min but declined gradually thereafter (Fig. 2A). LPS-induced NF-κB activation was suppressed in the presence of the NF-κB inhibitor, PDTC, at a concentration of 50 or 100 μM (Fig. 2B). The

protein production of TNF-α and IL-1β induced by LPS was also inhibited by pretreatment with PDTC in a dose-dependent manner (Fig. 2C and D). These data suggest that the production of TNF-α and IL-1β by LPS may be regulated by NF-κB at the transcriptional level.

Effects of xylitol on LPS-induced gene expression and protein synthesis of TNF-α and IL-1β. To clarify the effect of xylitol on LPS-induced TNF-α and IL-1β mRNA expression, RAW 264.7 cells were pretreated with 1, 2, 4, or 8% of xylitol 30 min prior to treatment with 1 μg of LPS/ml for 1 and 4 h, respectively. mRNA expression was examined by RT-PCR. Pretreatment with xylitol inhibited LPS-induced TNF-α and IL-1β mRNA expression in a dose-dependent manner. High doses of xylitol (4 or 8%) significantly inhibited TNF-α and IL-1β mRNA expression (Fig. 3A and B). Xylitol also inhibited TNF-α and IL-1β protein production increased by LPS in a dose-dependent manner (Fig. 3C and D). To rule out the hypothesis of toxicity of xylitol, cytolytic effects of xylitol were determined by measuring the cell viability by trypan blue exclusion. The xylitol concentrations used in the present study had no cytolytic effect on RAW 264.7 cells (data not shown).

Inhibition of LPS-induced NF-κB activation by xylitol. To investigate the effect of xylitol on LPS-induced NF-κB activation, RAW 264.7 cells were pretreated with 1, 2, 4, or 8% of
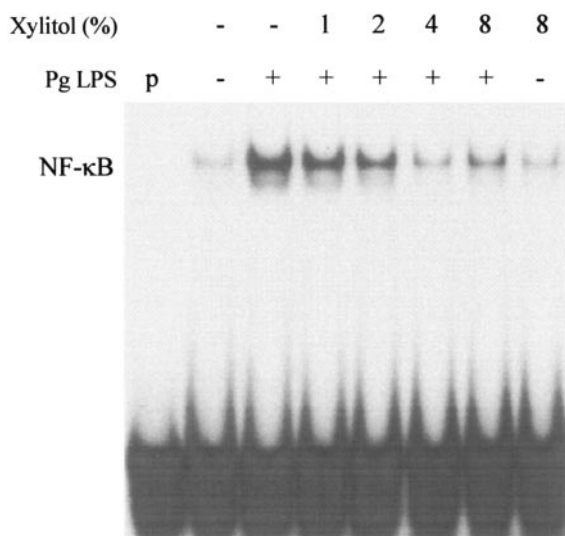
FIG. 4. Effect of xylitol on LPS-induced NF-κB activation. RAW 264.7 cells were pretreated with various concentrations of xylitol 30 min prior to treatment with 1 μg of LPS/ml. Nuclear extracts were prepared 30 min after LPS treatment, incubated with α-^{32}P-labeled κB oligonucleotide, and electrophoresed on a 4% polyacrylamide gel. Lane p contained probe incubated without extract.
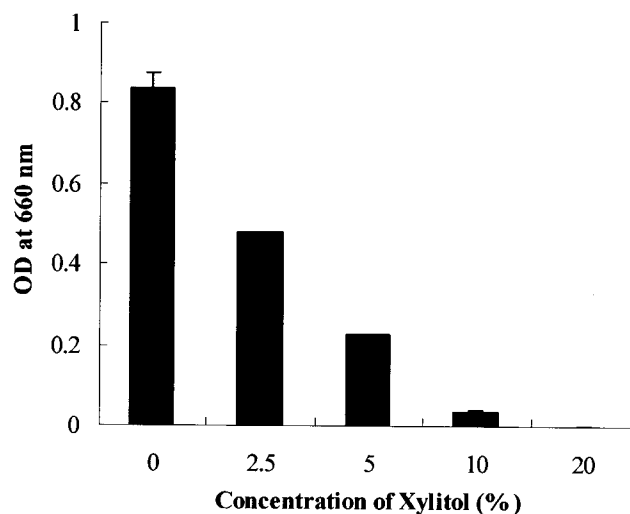

FIG. 5. Inhibitory effect of xylitol on the growth of *P. gingivalis*. *P. gingivalis* was cultured with various concentrations of xylitol for 24 h in anaerobic conditions. The growth of *P. gingivalis* was measured by determining the optical density at 660 nm with a spectrophotometer.

xylitol 30 min prior to treatment with 1 μg of LPS/ml. NF-κB activation was examined by gel shift assay. LPS enhanced NF-κB activation, and coincubation of LPS with increasing concentrations of xylitol resulted in a gradual decrease of NF-κB activation (Fig. 4). These data indicate that the inhibitory effect of xylitol on the production of inflammatory cytokine is mediated by inhibition of NF-κB activation.

Inhibitory effect of xylitol on the growth of *P. gingivalis*. Since it has been well known that xylitol inhibits the growth of mutans streptococci (45), the effect of xylitol on the growth of *P. gingivalis* was investigated. *P. gingivalis* was cultured for 24 h in anaerobic conditions with various concentrations of xylitol (0, 2.5, 5, 10, or 20%) and was measured at an optical density at 660 nm using a spectrophotometer. The growth of *P. gingivalis* was inhibited in a dose-dependent manner and entirely blocked in medium containing 20% xylitol. This result indicates that xylitol can have an inhibitory effect on the pathogenesis of *P. gingivalis* by inhibition of the growth of *P. gingivalis*, as well as inhibition of inflammatory cytokine production.

DISCUSSION

Periodontitis is a bone disease that impacts human health and economics. *P. gingivalis* has been predominantly isolated from subgingival plaques and is thought to be a major etiologic agent in adult periodontitis. Although the etiology of this disease is unknown, an increase in proinflammatory cytokine expression has been reported in pathology (5, 17, 22, 41). Xylitol have been used to prevent dental caries for the past two decades (24, 32). Xylitol inhibits the growth of oral microorganisms, especially *S. mutans*, one of the primary causative agents of dental caries (45). However, the effect of xylitol on an individual who has periodontal disease has not yet been investigated.

The present study demonstrates that (i) *P. gingivalis* LPS stimulates gene expression and the protein production of TNF-α and IL-1β, (ii) NF-κB is activated by LPS, (iii) xylitol inhibits LPS-induced gene expression and protein synthesis of TNF-α and IL-1β, (iv) xylitol inhibits LPS-induced NF-κB activation, and (v) xylitol inhibits the growth of LPS.

P. gingivalis elicits gingival tissue destruction and alveolar bone resorption. Bacterial components of *P. gingivalis*, such as LPS, have also been demonstrated to have the ability to activate osteoclast formation indirectly by stimulating osteoclasts or inflammatory cells as a consequence of inducing inflammatory cytokines (9, 30). Among inflammatory cytokines, IL-1 and TNF-α are thought to be pivotal factors in bone remodeling both in vitro and in vivo, and IL-6 has been observed in inflamed periodontal tissues (1, 2, 8, 10, 16, 18). Diseased gingival tissue expressed high levels of IL-1, TNF-α, and IL-1 receptor antagonist mRNA in the connective tissue compared to healthy gingival tissue (27, 34). Similarly, expression of mRNA for IL-6, IL-10, and IL-13 was observed from locally and chronically inflamed gingival of patients with adult periodontitis (48). The present study showed that LPS from *P. gingivalis* led to induce mRNA expression and protein production of TNF-α and IL-1β in mouse macrophage cell line, RAW 264.7 cells (Fig. 1). Because the nature and the role of the broad array of proinflammatory cytokines in the pathogenesis of periodontal disease are still poorly understood, further studies are needed to determine whether other resorption-stimulating factors are involved, as well as to establish the precise roles of members of the cytokine network in the periodontal pathology.

NF-κB is a multiunit transcription factor that plays a central role in induction of genes for proinflammatory cytokines (6, 11, 13, 36, 49) and many other immunoregulatory genes (3, 33). Thus, we investigated the possible involvement of NF-κB in LPS-induced TNF-α and IL-1β expression in RAW 264.7 cells. LPS was a potent inducer of NF-κB, and this LPS-induced

NF-κB activation and proinflammatory cytokine expression were suppressed by PDTC, an NF-κB inhibitor (Fig. 2). These data suggest that LPS induces TNF-α and IL-1β gene expression through the activation of NF-κB.

Xylitol is an artificial sweetener that has been successfully used in chewing gums to prevent dental caries (25, 38). It has been used as an oral sugar substitute without significant adverse effect (23). However, there are no studies examining the effects on the cells consisted of gingival tissues from patients with chronic periodontitis. Our present study demonstrated that xylitol is able to inhibit LPS-induced TNF-α and IL-1β gene expression and protein synthesis (Fig. 3) and that it is also able to inhibit LPS-induced NF-κB activation by pretreatment with xylitol (Fig. 4). Furthermore, xylitol directly inhibited the growth of *P. gingivalis* (Fig. 5). These data suggest that the antiperiodontitis effect of xylitol could be obtained from dual mechanisms. One is from the inhibitory effect of xylitol on the production of inflammatory cytokines, which is regulated at the transcriptional level by NF-κB. The other is that the growth of *P. gingivalis* is inhibited when it is cultured with xylitol. The inhibitory effect of xylitol on the growth of mutans streptococci has been widely known (21, 39, 46). The mechanism of xylitol on impaired growth of mutans streptococci is suggested to be an intracellular accumulation of xylitol 5-phosphate, and this xylitol 5-phosphate inhibits the activity of bacterial glycolytic enzymes (43).

Taken together with these data, xylitol may be a promising candidate as both an anticaries and an antiperiodontitis agent. We have shown here for the first time that xylitol can inhibit the periodontopathic effects induced by *P. gingivalis* LPS. To confirm the property of xylitol as an antiperiodontitis agent, further studies of xylitol on other inflammatory cytokines and other related factors are required.

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